Tumor Inhibitors. XLIV.[1a] The Isolation and Characterization of Hellebrigenin 3-Acetate and Hellebrigenin 3,5-Diacetate, Bufadienolide Tumor Inhibitors from *Bersama abyssinica*[1b]

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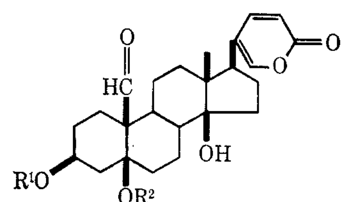
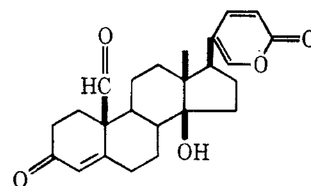
An alcoholic extract of the stem bark of *Bersama abyssinica* Fresen. was found to show significant inhibitory activity against human carcinoma of the nasopharynx in cell culture (KB). Systematic fractionation led to the isolation and characterization of hellebrigenin 3-acetate (1) and hellebrigenin 3,5-diacetate (2) as the major cytotoxic principles. Hellebrigenin 3-acetate was characterized by comparison with a sample prepared from hellebrigenin. Preliminary characterization of the 3,5-diacetate on the basis of spectral evidence indicated the presence of the bufadienolide skeleton and a second (tertiary) acetate group. The compound was shown to be a hellebrigenin diacetate by synthesis from hellebrigenin by acetylation with isopropenyl acetate and *p*-toluenesulfonic acid. The 3,5-diacetate structure was indicated by comparative studies of base-catalyzed ester solvolysis of 1 and 2, and confirmed by conversion of 2 to the methyl isohellebrigeninate derivative (6). A new and efficient enzymatic hydrolysis of hellebrin to hellebrigenin was developed, and a minor hydrolytic transformation product was assigned the scilliglaucosidin-3-one (4) structure. Hellebrigenin 3-acetate was found to show significant inhibitory activity against the Walker intramuscular carcinosarcoma 256 in rats, and is thus the first cardiotonic steroid found to show significant activity against an *in vivo* tumor system. Speculative rationalization of this observation is discussed.

In the course of a continuing search for tumor inhibitors from plant sources, an alcoholic extract of the stem bark of *Bersama abyssinica* Fresen. (Melianthaceae)[3] was found to show significant inhibitory activity against cells derived from human carcinoma of the nasopharynx carried in cell culture (KB).[4] Consequently, a systematic study aimed at isolation of the KB-inhibitory principles was undertaken. We report here in detail the systematic fractionation of the active extract of *B. abyssinica* Fresen. and the isolation and characterization of the major cytotoxic principles, hellebrigenin 3-acetate (1) and hellebrigenin 3,5-diacetate (2).[5,6]

The dried ground stem bark of *B. abyssinica* was extracted continuously with ethanol for 10 hr. Partition of the concentrated ethanolic extract (A) between water and chloroform resulted in concentration of the activity in the chloroform phase (C). The residue from the chloroform layer was defatted by partitioning between 10% aqueous methanol and petroleum ether and the activity was concentrated in the aqueous methanol layer (D). The material recovered by evaporation of the aqueous methanol layer was dissolved in methanol and treated with a saturated methanolic solution of neutral lead acetate. Removal of the precipitate by centrifugation and of the excess of lead with hydrogen sulfide gave the active extract (G) (see Chart I for summary and Table I for the cytotoxicity of the fractions). A



1, $R^1 = Ac; R^2 = H$
2, $R^1 = R^2 = Ac$
3, $R^1 = R^2 = H$



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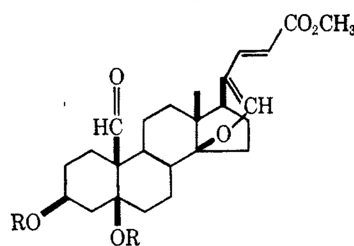

5, R = H
6, R = Ac

chloroform solution of fraction G was chromatographed on silicic acid, and fractions H–N were collected and examined by tlc. The major activity was eluted in two separate fractions (J and K), using 2% methanol in chloroform as eluant. Further careful rechromatography on silicic acid of fractions J and K separately led to the isolation of two crystalline compounds [hellebrigenin 3,5-diacetate (2) from fraction J and hellebrigenin 3-acetate (1) from fraction K] which accounted for the major cytotoxic activity of the original extract.

Compound 1 was identified as hellebrigenin 3-acetate by comparison of its physical constants with those

(1) (a) Part XLIII: S. M. Kupchan, A. P. Davies, S. J. Barboutis, H. K. Schnoes, and A. L. Burlingame, *J. Org. Chem.*, **34**, 3888 (1969). (b) Supported by grants from the National Cancer Institute (CA-04500) and the American Cancer Society (T-275), and a contract with Chemotherapy, National Cancer Institute, National Institutes of Health (PH 43-64-551).

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(3) Stem bark gathered in Ethiopia in March 1965. The authors acknowledge the receipt of the dried plant material from Dr. Robert E. Perdue, Jr., U. S. Department of Agriculture (USDA), Beltsville, Md., in accordance with the program developed with the USDA by the Cancer Chemotherapy National Service Center (CCNSC).

(4) Assays were performed under the auspices of the CCNSC. The procedures were those described in *Cancer Chemotherapy Rept.*, **25**, 1 (1962).

(5) A preliminary report of part of this work has been published: S. M. Kupchan, R. J. Hemingway, and J. C. Hemingway, *Tetrahedron Lett.*, 149 (1968).

(6) J. A. Lock [*J. Pharm. Pharmacol.*, **14**, 596 (1962)] has reported the presence of bufadienolides in the leaves of *B. abyssinica*, but the compounds were not characterized further.

CHART I

FRACTIONATION OF TUMOR-INHIBITORY EXTRACT FROM *B. abyssinica* Fresen.

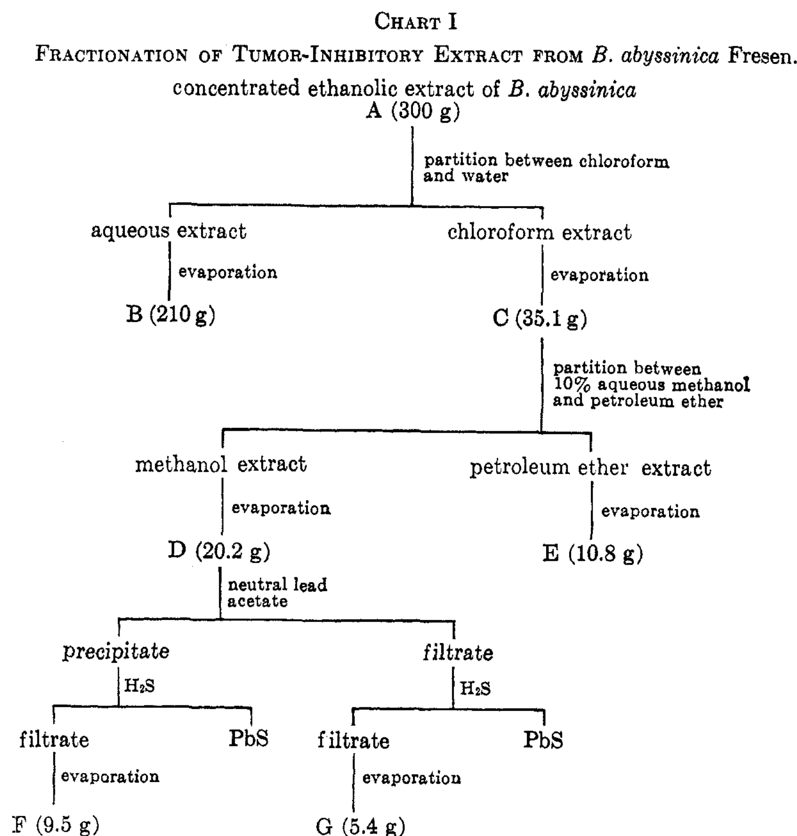

TABLE I

ACTIVITY OF FRACTIONS FROM *B. abyssinica* AGAINST KB TISSUE CULTURE

Fraction	ED_{50}, μg/ml	Fraction	ED_{50}, μg/ml
A	2.0	Q	19
B	8.1	R	1.3×10^{-3}
C	0.22	S	2.2×10^{-2}
D	0.33	T	0.17
E	>100	U	19
F	1.7	V	0.15
G	0.12	W	1.3×10^{-5}
H	2.7	X	4.9×10^{-6}
		Y	2.3×10^{-2}
J	8.9×10^{-4}	Z	2.8×10^{-2}
K	2.2×10^{-2}	AA	0.21
L	7.8×10^{-2}		
M	0.17	**1**	2.4×10^{-7}
N	2.5	**2**	1.9×10^{-3}
O	17	**3**	5.9×10^{-3}
P	23		

TABLE II

NUCLEAR MAGNETIC RESONANCE DATA[a]

Compd	C-3	C-18	C-19	C-21	C-22	C-23	–OAc
1[b]	4.76 m	9.32 s	0.00 s	2.76 dd (2, 1)	2.20 dd (2, 9.5)	3.75 dd (9.5, 1)	7.92 s
2[c]	4.84 m	9.32 s	0.00 s	2.76 dd (2, 1)	2.20 dd (2, 9.5)	3.75 dd (9.5, 1)	8.01 s, 8.05 s
4[d]	...	9.25 s	0.05 s	2.75 dd (2, 1)	2.20 dd (2, 9.5)	3.73 dd (9.5, 1)	...
5[e]	5.82 m	9.02 s	−0.08 s	3.43 s	2.77 d (15)	4.38 d (15)	...
6[f]	4.85 m	9.05 s	−0.20 s	3.44 s	2.80 d (15)	4.38 d (15)	7.99 s, 8.02 s

[a] Spectra were determined on a Varian A-60A spectrometer in deuteriochloroform solutions. Values are given in τ units relative to tetramethylsilane as internal standard. Multiplicity of signals is designated as follows: s, singlet; dd, doublet of doublets; m, multiplet; br m, broad multiplet. Numbers in parentheses denote coupling constants in hertz. [b] 23 additional protons, τ 7.0–9.0 br m. [c] 22 additional protons, τ 7.0–9.0 br m. [d] C-4 H, τ 4.00 s, 20 additional protons, τ 7.0–9.0 br m. [e] –OMe τ 6.25 s, –OH τ 5.8 m, 22 additional protons, τ 7.0–9.0 br m. [f] –OMe τ 6.25 s, 21 additional protons, τ 7.0–9.0 br m.

reported in the literature,[7] and by direct comparison with an authentic sample of hellebrigenin 3-acetate prepared by acetylation of hellebrigenin. This appears to be the first report of the isolation of hellebrigenin 3-acetate from a natural source. Hellebrigenin 3-acetate was highly cytotoxic (see Table I) and only those column fractions of K which contained **1** showed marked cytotoxic activity.

Hellebrigenin 3,5-diacetate (**2**) was assigned the formula $C_{28}H_{36}O_8$ on the basis of elemental analysis. The infrared spectrum exhibited bands at 5.80, 6.12, and 6.50 μ and the ultraviolet spectrum showed λ_{max}^{MeOH} 298 mμ (ε 5,650), typical of the α-pyrone ring of a bufadienolide. The infrared spectrum also showed peaks at 2.90 (hydroxyl), 3.38, 5.80 (complex carbonyl), and 7.95 μ (acetate). The nmr spectrum provided further evidence for the cited functional groups, and comparison of the nmr spectra of **1** and **2** (see Table II) supported the view that **2** was a diacetate of hellebrigenin. The nmr spectrum of **2** exhibited only one CHOAc proton, and indicated that the second acetate must be tertiary and thus located either at C-5 or at C-14. To test the hypothesis that the compound was, indeed, a diacetate derivative of hellebrigenin, the synthesis of **2** from hellebrigenin was undertaken.

An attractive potential source of hellebrigenin is the glycoside hellebrin, which has previously been hydrolyzed to hellebrigenin by a two-step sequence in an overall yield of about 50%.[8] In an attempt to

(7) J. Schmutz, *Helv. Chim. Acta*, **32**, 1442 (1949). We thank Professor T. Reichstein cordially for an authentic sample of hellebrigenin 3-acetate.

(8) J. Schmutz, *Pharm. Acta Helv.*, **22**, 373 (1947).

improve upon the hydrolytic procedure, we investigated the hydrolysis of hellebrin[9] with a Clarase-concentrate enzyme preparation[10] in a phosphate buffer. The solution was incubated at 37° for several days with rapid stirring, and the reaction was followed by tlc. After 10 days, hydrolysis of hellebrin was complete. During the course of the reaction, the solution became cloudy owing to bacterial growth. The bacteria were present in both batches of enzyme used and appeared to be essential for the hydrolysis reaction, since, when steps were taken to prevent bacterial growth, no hellebrigenin was obtained. The bacterial contaminant was identified as a *Pseudomonas*.[11] When hydrolysis was complete, the aqueous solution was extracted with chloroform which was evaporated, and the residue was crystallized to give hellebrigenin in 80% yield.

Toward the end of the hydrolysis, the formation of a new material with higher R_f than hellebrigenin was observed. This product was isolated from the mother liquors after the crystallization of hellebrigenin and shown to be a new bufadienolide derivative, scilliglaucosidin-3-one (**4**). The structural assignment was first suggested on the basis of the mass spectrum, which exhibits m/e 396 (M^+), 378 (M − 18), and 368 (M − 28) (but no peak corresponding to further loss of hydroxyl), which indicated a molecular formula formally derived from hellebrigenin by loss of 2 H and H_2O. Confirmation was obtained from the infrared, ultraviolet, and nmr spectra. The infrared spectrum exhibited strong absorption at 6.01 μ and the ultraviolet spectrum showed an additional maximum at 245 mμ, typical of an α,β-unsaturated ketone. The nmr spectrum exhibited a singlet at τ 4.00 corresponding to the vinyl proton at C-4. Corroboration of the spectral evidence was obtained by synthesis of scilliglaucosidin-3-one from scilliglaucosidin by oxidation with manganese dioxide. A precedent for a microbiological transformation of this type has been reported, in which the analogous cardenolide, strophanthidin, was converted by the fungus *Chaetomium globulosum* to anhydrostrophanthidone.[12]

Hellebrigenin was acetylated in chloroform with isopropenyl acetate and a catalytic amount of *p*-toluenesulfonic acid. The major product was hellebrigenin 3-acetate (**1**); the minor product, isolated by chromatography, was **2**. With longer reaction periods, complex mixtures were obtained with increased proportions of less polar products and no increase in the yield of **2**. When the reaction was terminated after 3–4 hr, the isolated hellebrigenin 3-acetate could be recycled to yield additional diacetate. The results demonstrated that both hellebrigenin and hellebrigenin 3-acetate could be converted to the diacetate, and that the structural possibilities could therefore be limited to hellebrigenin 3,5-diacetate or 3,14-diacetate.

Characterization of the diacetate as hellebrigenin 3,5-diacetate was made possible by a study of the behavior of **1** and **2** under conditions which favor base-catalyzed ester solvolysis. Earlier detailed studies had shown that the ready solvolysis of the 3-axial acetate in strophanthidin 3-acetate is a general acid–general base catalyzed reaction, which is facilitated both by the C-5 hydroxyl group bearing a 1,3-diaxial juxtaposition to the acetate and by the C-19 aldehyde function.[13] Hellebrigenin 3-acetate differs from strophanthidin 3-acetate only in the lactone ring, and, in accord with expectation, treatment of **1** in 20% aqueous methanol with triethylamine at room temperature for 16 hr led to essentially complete solvolysis to hellebrigenin. In contrast, treatment of **2** under the same conditions effected no solvolysis, and **2** was recovered essentially quantitatively. The stability of **2** toward base-catalyzed solvolysis indicated the absence of a free 5β-hydroxyl group in **2** and, therefore, strongly favored assignment of the hellebrigenin 3,5-diacetate structure.

Confirmation of the hellebrigenin 3,5-diacetate structure for **2** was achieved by demonstrating that both the diacetate and hellebrigenin readily formed isohellebrigeninic acid derivatives. Treatment under conditions described earlier for other bufadienolides[14] led to conversion of hellebrigenin to methyl isohellebrigeninate (**5**) and of the diacetate to methyl isohellebrigeninate 3,5-diacetate (**6**). Compound **2** is therefore hellebrigenin 3,5-diacetate and appears to be the first reported 5β-acetoxy steroid derivative.

The results of assays of hellebrigenin (**3**), the 3-acetate **1**, and the 3,5-diacetate **2** for cytotoxicity against KB have been listed in Table I, and the results of assays for *in vivo* activity against the Walker intramuscular carcinosarcoma 256 in rats have been listed in Table III. Although other cardenolides[15–18] and bufadienolides[17] have been found earlier to possess marked cytotoxic activity against KB tissue culture, hellebrigenin 3-acetate appears to be the first cardiotonic steroid recognized to show significant activity against an *in vivo* tumor system.

A parallelism between cytotoxic activity and in-

TABLE III

ASSAYS AGAINST WALKER INTRAMUSCULAR CARCINOSARCOMA 256[a,b]

Compd	Dose, mg/kg	Survivors	Animal wt change difference, g, T − C	Tumor wt, mg T/C	T/C × 100
1	10	1/4	−21	1000/4800	. . .
	8	4/4	−16	1200/4800	25
	6	3/4	−18	900/4800	18
	4	4/4	−10	2900/4800	60
2					Inactive
3					Inactive

[a] Reference 4. [b] T, treated animals; C, control animals.

(9) We thank Dr. A. Brossi, Hoffmann-La Roche and Co., for a generous gift of hellebrin.

(10) We thank Miles Laboratories, Inc., Clifton, N. J., for the samples of Clarase (Concentrate, Control No. F1407).

(11) We thank Professor D. Perlman cordially for suggesting the use of the Clarase enzyme preparation and for identifying the bacterial contaminant.

(12) C. J. Sih, S. M. Kupchan, O. El Tayeb, and A. Afonso, *J. Med. Chem.*, **5**, 629 (1962).

(13) S. M. Kupchan, S. P. Eriksen, and M. Friedman, *J. Amer. Chem. Soc.*, **84**, 4159 (1962); **88**, 343 (1966).

(14) A. Stoll, A. von Wartburg, and J. Renz, *Helv. Chim. Acta*, **36**, 1531 (1953).

(15) S. M. Kupchan, R. J. Hemingway, and R. W. Doskotch, *J. Med. Chem.*, **7**, 803 (1964).

(16) S. M. Kupchan, J. R. Knox, J. E. Kelsey, and J. A. Saenz Renauld, *Science*, **146**, 1685 (1964).

(17) R. B. Kelly, E. G. Daniels, and L. B. Spaulding, *J. Med. Chem.*, **8**, 547 (1965).

(18) S. M. Kupchan, M. Mokotoff, R. S. Sandhu, and L. E. Hokin, *ibid.*, **10**, 1025 (1967).

hibition of transport ATPase with different cardenolides has been noted earlier.[18] Furthermore, the active transport of many amino acids is inhibited by cardenolides, possibly because of an interrelationship between amino acid transport and sodium transport.[19] Inasmuch as tumor cells are very active in accumulating amino acids,[20] it is possible that the inhibition in growth of tumor cells by cardiotonic steroids is due to inhibition of amino acid accumulation. A possible rationalization of the observation of *in vivo* tumor-inhibitory activity of bufadienolide **1** may lie in the recently noted apparent divergence between relative activities of butadienolides and cardenolides with regard to cardiotonic activity, on the one hand, and ATPase-inhibitory activity, on the other.[21] The observed *in vivo* activity of **1** may be attributable to a marked effectiveness in inhibiting amino acid accumulation by tumor cells when administered in doses well below those which are toxic to the heart.

Experimental Section

Melting points were determined on a Thomas-Hoover capillary melting point apparatus and are corrected. Infrared spectra were determined on Beckman Models 5A and 9 recording spectrophotometers. Ultraviolet absorption spectra were determined on a Beckman Model DK-2A recording spectrophotometer. Specific rotations were determined on a Zeiss-Winkel polarimeter and are approximated to the nearest degree. Petroleum ether refers to the fraction with bp 60-68°. Evaporations were carried out under reduced pressure at temperatures of less than 40°. Thin layer chromatography was carried out on silica gel (E. Merck) plates and chromatograms were sprayed with a 3% ceric sulfate in 3 *N* sulfuric acid solution followed by heating. Microanalyses were carried out by Spang Microanalytical Laboratory, Ann Arbor, Mich.

Extraction and Preliminary Fractionation of *Bersama abyssinica*.—Coarsely ground stem bark of *B. abyssinica* (2.2 kg) was twice extracted continuously with 95% ethanol for 5 hr and the ethanol extract was concentrated under reduced pressure to a dark semisolid (A, 300 g). The extract was partitioned by shaking vigorously with water (1.5 l.) and chloroform (5 l.), and the mixture was allowed to separate slowly over several hours. The two solutions were evaporated to yield B (210 g) and C (35.1 g), respectively. The chloroform solubles (C) were partitioned between 10% aqueous methanol (300 ml) and petroleum ether (600 ml) and the two solutions were evaporated to yield D (20.2 g) and E (10.8 g), respectively. A solution of the 10% aqueous methanol solubles (D) in methanol (200 ml) was treated with a saturated methanolic solution of neutral lead acetate to complete precipitation. The precipitate was separated by centrifuging, washed with methanol, suspended in methanol, and treated with hydrogen sulfide. The lead sulfide was removed by filtration and the filtrate was evaporated to give F (9.5 g). The supernatant liquid from the lead acetate precipitation was also treated with hydrogen sulfide, the lead sulfide was filtered, and the filtrate was evaporated to give G (5.4 g).

Isolation of Bufadienolides.—A larger batch of fraction G (68.04 g) was prepared from the crude extract (3.48 kg) and a portion (23 g) was further fractionated by chromatography on silicic acid (2.2 kg). The column was eluted with chloroform (15 l.) and 1% methanol in chloroform (10 l.) until the eluate was colorless. The solvent was changed to 2% methanol in chloroform (20 l.). Fractions were collected and examined by tlc using 7% methanol in chloroform as solvent and ceric sulfate spray reagent, when, on warming slowly, the active materials were visualized as green spots. All fractions eluted before a green spot were combined with the chloroform and 1% methanol in chloroform eluates and evaporated under reduced pressure to yield H (9.10 g). Fractions containing a high R_f green spot were combined and evaporated to yield J (1.13 g). Fractions containing a lower R_f green spot were combined (K, 1.98 g). The remaining fractions were combined (L, 3.07 g). The solvent was changed to 5% methanol in chloroform (8 l.) to give M (1.17 g). The remaining material (N, 6.15 g) was removed with methanol. A larger batch of fractions J (1.87 g) and K (2.14 g) was prepared from the remaining fraction G.

Fraction J (2.50 g) was dissolved in chloroform and added to a column of silicic acid-Celite (300:50 g). The column was eluted with chloroform (4 l.) and the solvent was evaporated (residue, 78 mg). The solvent was changed to 1% methanol in chloroform, and the fractions (20 ml) were collected and examined by tlc on silica gel G using 7% methanol in chloroform as solvent and ceric sulfate spray reagent. The fractions were combined and evaporated as follows: 1-124, P (200 mg), no significant spot on tlc; 125-147, Q (347 mg), brown spot on tlc; 148-164, R (870 mg), high R_f green spot on tlc; 165-204, S (334 mg), all fractions after green spot; methanol wash, T (352 mg).

Upon crystallization of fraction R from methanol-ether, two solid materials separated. The white flocculent material was removed by decantation to leave colorless prisms. Recrystallization from methanol gave colorless prisms of hellebrigenin 3,5-diacetate (**2**, 45 mg): mp 217-219°; $[\alpha]^{28}D$ −23° (*c* 0.51, $CHCl_3$); uv λ_{max}^{MeOH} 298 mμ (ϵ 5,650); ir $\lambda_{max}^{CHCl_3}$ 2.90, 3.38, 5.80, 6.12, 6.50, and 7.95 μ.

Anal. Calcd for $C_{28}H_{36}O_8$: C, 67.18; H, 7.25. Found: C, 67.15; H, 7.38.

Fraction K (2.90 g) was dissolved in chloroform and added to a column of silicic acid (260 g). The column was eluted with chloroform (4 l.) and the solvent was evaporated to give U (74 mg). The solvent was changed to 1% methanol in chloroform and fractions (20 ml each) were collected and examined by tlc on silica gel G using 7% methanol in chloroform as solvent and ceric sulfate spray reagent. Fractions were combined and evaporated as follows: 1-145, V (186 mg), white spot on tlc; 146-162, W (913 mg), low R_f green spot; 163-190, X (420 mg), low-R_f green spot; 191-250, Y (398 mg), fractions after green spot; 2% eluate (4 l.), Z (60 mg); methanol wash, AA (318 mg).

Fraction W was crystallized from methanol to yield colorless prisms (164 mg) of hellebrigenin 3-acetate (**1**): mp 230-232°; $[\alpha]^{30}D$ +30° (*c* 1.25, $CHCl_3$). Fraction X was crystallized from methanol to yield an additional sample (175 mg) of **1**. An earlier report[7] cited the physical constants mp 242-247°; $[\alpha]D$ +33.7° (*c* 0.74, $CHCl_3$). The melting point of **1** was not depressed by admixture with an authentic sample.[7] The two samples showed identical R_f values on tlc and superimposable ir spectra ($CHCl_3$).

Hydrolysis of Hellebrin. A. Hellebrigenin (3).—Hellebrin (2.5 g), Clarase concentrate (2.5 g), and potassium dihydrogen phosphate (17.5 g) were dissolved in distilled water (2.5 l.). The solution was warmed to 37° and stirred rapidly for 10 days. A sample of the solution, upon evaporation to dryness, showed a major spot corresponding to hellebrigenin on tlc with silica gel G, 12% methanol in chloroform as solvent, and ceric sulfate spray reagent. The aqueous solution was extracted with chloroform (4 l.). The chloroform extracts were dried (Na_2SO_4) and evaporated to yield a pink solid (1.35 g). Crystallization from acetone-ether yielded colorless prisms of **3** (1.15 g): mp 225-227°; $[\alpha]^{26}D$ +19° (*c* 0.83, acetone) [lit.[22] mp 150-153°, 237-240°, or 250-253°; $[\alpha]D$ +17.8° (acetone)].

B. Scilliglaucosidin-3-one (4).—The mother liquor (1.2 g) from the crystallization of hellebrigenin was chromatographed on silica gel (350 g) with methanol-acetone-chloroform (1:4:15) as solvent. Fractions were combined on the basis of tlc and evaporated. The major fraction was crystallized from acetone-ether to give colorless prisms (480 mg) of **3**, mp 235-236°. The minor fraction was crystallized from chloroform-ether to give colorless prisms (65 mg), mp 268-270° dec. Recrystallization from methanol gave crystals of **4**: mp 278-279° dec; uv λ_{max}^{MeOH} 245 (ϵ 12,100) and 299 mμ (ϵ 5, 170); ir λ_{max}^{KBr} 2.81, 2.88, 3.40, 3.49, 5.93, 6.01, 6.12, and 6.50 μ.

Anal. Calcd for $C_{24}H_{28}O_5 \cdot {}^1/_2CH_3OH$: C, 71.33; H, 7.27. Found: C, 71.51; H, 7.30.

Manganese Dioxide Oxidation of Scilliglaucosidin.—A solution of scilliglaucosidin (22 mg) in chloroform (5 ml) was stirred with manganese dioxide (100 mg) at 35° for 4 hr, and the mixture was chromatographed on silicic acid (4 g). The fractions eluted with 1% methanol-chloroform were combined on the basis of tlc and

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(20) H. N. Christensen, T. R. Riggs, H. Fischer, and I. M. Palatine, *J. Biol. Chem.*, **198**, 1 (1952).

(21) L. E. Hokin, J. L. Dahl, M. H. Kline, and S. M. Kupchan, unpublished observations.

(22) C. Tamm, *Progr. Chem. Org. Nat. Prod.*, **14**, 71 (1957).

evaporated. The residue was crystallized from chloroform–hexane to give colorless prisms of **4** (4 mg), mp 266–269° dec, characterized by melting point, mixture melting point, tlc, and ir spectral comparison with **4** prepared from hellebrin.

Acetylation of Hellebrigenin.—A solution of hellebrigenin (510 mg) and *p*-toluenesulfonic acid (10 mg) in a mixture of chloroform (5 ml) and isopropenyl acetate (10 ml) was stirred for 3 hr at 45–50°. The solution was evaporated and the residue was chromatographed on silicic acid (40 g). Elution with 0.5% methanol in chloroform gave a fraction (32 mg) rich in **2**. Elution with 1% methanol in chloroform gave a fraction (333 mg) rich in **1**. The latter fraction was recycled, and the product again was chromatographed on silicic acid to yield fractions (44 mg and 245 mg) rich in **2** and **1**, respectively. A second recycling of the 245-mg fraction yielded a further fraction (45 mg) rich in **2**. The combined fractions rich in **2** (121 mg) were chromatographed on silica gel (20 g). Elution with acetone–methanol–chloroform (10:2:88) yielded a fraction (99 mg) which was crystallized from methanol–ether as colorless prisms of hellebrigenin 3,5-diacetate (**2**, 30 mg), mp 209–214°. The melting point of this compound was not depressed by admixture with the sample from *B. abyssinica*. The two samples showed identical R_f values on tlc and gave superimposable ir spectra ($CHCl_3$).

Solvolysis of Hellebrigenin 3-Acetate.—A solution of hellebrigenin 3-acetate (**1**, 10 mg) in 20% aqueous methanol (5 ml) was treated with triethylamine (50 mg). After standing at room temperature for 16 hr, the solution was concentrated under reduced pressure to a small volume, diluted with water (5 ml), and extracted with chloroform (20 ml). The chloroform extracts were dried (Na_2SO_4) and the solvent was evaporated to yield an essentially homogeneous (tlc) colorless oil (9 mg). Crystallization from acetone–ether yielded colorless prisms of hellebrigenin, mp 225–227°, characterized by mixture melting point and tlc.

Attempted Solvolysis of Hellebrigenin 3,5-Diacetate.—A solution of hellebrigenin 3,5-diacetate (**2**, 9 mg) in 20% aqueous methanol (5 ml) was treated with triethylamine (50 mg) in the same manner as described for **1**, to yield an essentially homogeneous (tlc) colorless residue (9 mg). Crystallization from methanol–ether led to recovery of hellebrigenin 3,5-diacetate as colorless prisms, mp 214–217°.

Methyl Isohellebrigeninate (5).—Hellebrigenin (110 mg) was treated with a solution of sodium hydroxide (250 mg) in methanol (10 ml). After standing for 4 hr at room temperature, the solution was treated with ice–water (30 ml) and acidified with hydrochloric acid. The precipitate was filtered, washed with water, and dried under reduced pressure. The residue (86 mg) was crystallized from acetone–hexane to give prisms (64 mg). Recrystallization from methanol gave colorless prisms of **5**: mp 235–240° dec; uv λ_{max}^{MeOH} 300 mμ (ϵ 21,100); ir λ_{max}^{KBr} 2.96, 3.39, 3.48, 3.63, 5.81, 5.86, 6.19, 6.22, and 8.59 μ.

Anal. Calcd for $C_{25}H_{34}O_7$: C, 69.74; H, 7.96. Found: C, 70.06; H, 7.88.

Methyl Isohellebrigeninate 3,5-Diacetate (6).—Hellebrigenin 3,5-diacetate (50 mg) was treated with a solution of sodium hydroxide (40 mg) in methanol (4 ml). After 1 hr, the product was worked up as described for methyl isohellebrigeninate and the residue (35 mg) was chromatographed on silicic acid (5 g). The fractions eluted with chloroform were combined on the basis of tlc to give an oil (15 mg). Crystallization from ether–hexane gave colorless plates of **6** (8.5 mg): mp 207–210°; uv λ_{max}^{MeOH} 300 mμ (ϵ 24,800); ir λ_{max}^{KBr} 2.90, 3.37, 3.48, 3.63, 5.76, 5.81, 5.85, 6.19, 6.21, 7.95, and 8.60 μ.

Anal. Calcd for $C_{29}H_{38}O_8$: mol wt, 514.25668. Found: mol wt, 514.25830 (mass spectrum).

Registry No.—**1**, 4064-09-9; **2**, 16808-82-5; **4**, 21887-06-9; **5**, 21887-07-0; **6**, 21904-42-7.

Tumor Inhibitors. XLV.[1a] Crotepoxide, a Novel Cyclohexane Diepoxide Tumor Inhibitor from *Croton macrostachys*[1b]

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An alcoholic extract of the fruits of *Croton macrostachys* Hochst. *ex* A. Rich. was found to show significant inhibitory activity against the Lewis lung carcinoma in mice (LL). The major active component, crotepoxide (**1**), $C_{18}H_{18}O_8$, was isolated and found to be a novel cyclohexane diepoxide. Alkaline hydrolysis and spectral evidence showed it to be the benzoate and diacetate derivative of the triol **2**. Hydrogenation of **1** reduced the aromatic ring to give a hexahydro derivative **3**. On treatment with hydrochloric acid, crotepoxide yielded initially the monochlorohydrin **4**, and then the dideacetyldichlorohydrin **5**. Treatment of **1** with hydriodic acid yielded the iodohydrin **8** and the ene diol **9**, whose reactivity has been studied. The structure and absolute stereochemistry of **8** were established by X-ray crystallographic analysis and, together with spectral evidence, were used to confirm the absolute stereochemistry and structure of **1**.

In the course of a continuing search for tumor inhibitors from plant sources, an alcoholic extract of the fruits of *Croton macrostachys* Hochst. *ex* A. Rich. (Euphorbiaceae)[3] was found to show significant inhibitory activity against Lewis lung carcinoma carried in mice (LL).[4] We report herein the systematic fractionation of the active extract of *C. macrostachys* and the isolation and structural elucidation of crotepoxide, the major active principle.

The dried ground fruits of *C. macrostachys* were extracted continuously with ethanol for 16 hr. Partition of the concentrated ethanolic extract (A) between 10% aqueous methanol and petroleum ether resulted in concentration of the activity in the methanol phase (C). The residue after evaporation of the methanol was partitioned between 1-butanol (D) and water (E) (Chart I and Table I). Fraction D in chloroform was chromatographed on silicic acid. The active component was eluted with chloroform, and rechromatography on silicic acid led to the isolation of crotepoxide, which showed significant tumor-inhibitory activity against Lewis lung carcinoma in mice (LL) and Walker intramuscular carcinosarcoma in rats (WM)[4] (Table I).

On the basis of elemental analysis, crotepoxide was assigned the molecular formula $C_{18}H_{18}O_8$. The pres-

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(3) Fruits gathered in Ethiopia in March 1965. The authors acknowledge the receipt of the dried plant material from Dr. Robert E. Perdue, Jr., U. S. Department of Agriculture (USDA), Beltsville, Md., in accordance with the program developed with the USDA by the Cancer Chemotherapy National Service Center (CCNCS).

(4) Assays were performed under the auspices of the CCNSC. The procedures were those described in *Cancer Chemotherapy Rept.*, **25**, 1 (1962).